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(54) **Diequitorially bound beta 1-4 polyuronates and use of same for cytokine stimulation.**

(57) The present invention is directed to a material and method for the stimulation of the production of cytokines. Several polysaccharides, including polymers of different size of  $\beta$ 1-4 linked D-mannuronic acid (poly-M and M-blocks), chitosan and cellulose oxidized in C-6 position (C60XY) induce human monocytes to produce the cytokines. Preferably, the molecular weights of poly-M and chitosan are above 50,000 and 20,000 respectively. Pretreatment of the monocytes with IFN- $\gamma$  increases the cytokine production from monocytes stimulated with all polysaccharides tested. The subject polysaccharides worked in vivo and in vitro.

The present invention has therapeutic utility as vaccine adjuvants and components. Therapeutic compositions comprising biologically active quantities of the compositions of the present invention may be employed to potentiate antibody production in response to vaccine antigens. Anti-tumor, anti-bacteriological, anti-fungal and anti-viral effects may be expected.

**BACKGROUND OF THE INVENTION****1. FIELD OF THE INVENTION:**

5 This invention relates to the fields of biochemistry and immunology. More specifically, this invention provides a method of in vivo and in vitro stimulation of the production of cytokines for various medicinal, biochemical and physiological effects.

**2. ART BACKGROUND:**

10 The lymphocytes or white blood cells in the animal body come in two types, B-cells and T-cells. The B-cells produce antibodies in the form of immunoglobulins that bind onto invading organisms while the T-cells produce the lymphokines or cytokines which are responsible for turning B-cells on or off. It is well known that the growth of normal lymphocytes is dependent not only on contact with an antigenic substance or a mitogen, but also on the presence of certain factors known as lymphokines or cytokines.

15 The known types of lymphokines include IL-2, B-cell factors, macrophage activation factor (MAF), Interleukin-3 (IL-3), Colony Stimulating Factor (CSF), Tumor Necrosis Factor (TNF), and other factors produced by monocytes such as Interleukin-1 (IL-1) and Gamma Interferon. All of these factors are secreted by white blood cells and are collectively known as cytokines.

20 Monocytes can be stimulated by various agents, such as LPS (lipopolysaccharides), to produce certain cytokines including TNF, IL-1 and IL-6. LPS contain a polysaccharide a lipid A part. Polysaccharides such as  $\beta$  1-3 glucans have been reported to stimulate monocytes to produce cytokines. Seljelid R., Figenschau Y., Bøgwald J., Rasmussen L.T., and R. Austgulen. 1989. Evidence that Tumour Necrosis Induced by Aminated  $\beta$  1-3D Polyglucose is Mediated by a Concerted Action of Local and Systemic Cytokines. Scand. J. Immunol., 30:687; Otterlei M., Østgaard K., Skjåk-Bræk G., Smidsrød O. and T. Espevik. 1991. Induction of cytokine production from human monocytes stimulated with alginate, J. of Immunotherapy, in press.

25 The present invention relates generally to cytokines, and particularly to the stimulation of biologically active mammalian IL-1, IL-6 and TNF. Interleukin-1 is the designation given to a family of polypeptides, released by macrophages and certain other cell types in response to immunogenic and traumatic stimulation, which have a primary role in initiating host response to injury and infection. These cytokines have been associated with a complex spectrum of biological activities. Specifically, IL-1 is a primary immunostimulatory signal capable of inducing thymocyte proliferation via induction of Interleukin-2 release, and of stimulating proliferation and maturation of B lymphocytes. In addition, IL-1 has been linked with prostaglandin production, induction of fever and promotion of wound healing.

30 In recent years, immunotherapy has become more and more important in the field of applied medicine. For instance opportunistic infections are frequently observed in newborn children with functional immunodeficiency, cancer patients, patients transplanted with bone marrows, patients receiving chemotherapeutic agents or steroids and aged patients. Antibiotics so far used in these field against infections do not exert significant effect in immuno-deficient or immuno-depressed patients. The situation is almost the same in viral infections, and effective chemotherapeutic agents against viral infections has not been so far provided for clinical uses. Interferon has been reported to have some effects against viral infections; however, the nature of the interferons is still unclear and there is uncertainty as to the action modes. Therefore, interferon has not yet been confirmed for its effectiveness. The uncertainty of the effectiveness of interferon is also due to the fact that human interferon does not function in animal model. The presents of species-specificity may be one of the major difficulty for the development of interferon as therapeutic agents.

35 In the field of cancer, the situation of the development of effective therapeutic drugs is analogous to those in infectious diseases and viral diseases. In cancer patients, the prominent depression of immunological function of the host has been widely confirmed, and therefore immunotherapy and immunochemotherapy (adjuvant therapy) have drawn much attention as new definite methods to treat cancer patients. Immunotherapy has been said not only to restore or potentiate the specific immune responses against cancer, but also to improve the general function of host including immunological functions. So far several immunomodulators have been applied in clinics, however almost all of these share the nature of chemotherapeutic agents in such a manner that they also exert direct cytotoxic effects against neoplastic cells.

40 Generally speaking, several antigens or immunogens containing the antigens are also used as vaccines in active immunotherapy. However the active immunotherapy does not function when the immunological functions of the host are depressed or defective such as in cancer, infections and viral diseases. In order to augment humoral immune responses, the administration of gamma -globulin together with the vaccines might be useful. However, when cell-mediated immunity play key roles, the methods is not practical. In such cases, cytokines

might be useful when used in combination with vaccines such as tumor specific antigens instead of gammaglobulin in the augmentation of humoral immune responses.

Additionally, research has demonstrated that IL-1, TNF, and other leukocyte derived cytokines are important, and even critical, mediators in a wide variety of inflammatory states and diseases. The immunostimulating activities of cytokines may be important for treatment of neoplastic as well as for infectious diseases, and may have potential anti-tumor, anti-bacteriological anti-fungal and antiviral effects. Additionally, therapeutic compositions comprising biologically active quantities of certain cytokines and derivatives could be employed to potentiate antibody production in response to vaccine antigens. Also, in view of potential therapeutic utility as vaccine adjuvants and components of wound-healing compositions, there is interest in employing cytokines in veterinary medicine.

## **SUMMARY OF THE INVENTION**

The present invention is directed to materials and methods for the stimulation of the production of cytokines. It has been found herein that several well-defined polysaccharides, including polymers of different size of  $\beta$ 1-4 linked D-mannuronic acid (poly-M) and cellulose oxidized in C-6 position (C60XY), and chitosan induces human monocytes to produce TNF- $\alpha$ . Poly-M was the most potent polysaccharide tested.

TNF- $\alpha$  production was shown to depend strongly on the molecular weight of poly-M and chitosan, with a maximal TNF- $\alpha$  production for a m.w. above 50,000 and 20,000 respectively.

Pretreatment of the monocytes with IFN- $\gamma$  increases the TNF $\alpha$  production from monocytes stimulated with all polysaccharide tested in a similar way as for monocytes stimulated with LPS. C60XY injected intravenously in mice led to rapid production of TNF $\alpha$  and IL-6.

In view of potential therapeutic utility as vaccine adjuvants and components of wound-healing compositions, the present invention may be employed as such an agent. Therapeutic compositions comprising biologically active quantities of the compositions of the present invention may be employed to potentiate antibody production in response to vaccine antigens. Anti-tumor, anti-fungal, anti-bacteriological and anti-viral effects may be expected.

The present invention relates to several polysaccharides that are capable of stimulating cytokine producing cells to produce significant quantities of cytokines both *in vivo* and *in vitro*. This discovery makes possible a novel therapeutic approach in the treatment of bacterial, fungal and viral infections, cancer, immune deficiencies and autoimmune diseases.

Accordingly, it is an object of the invention to provide pharmaceutical compositions that are useful as therapeutic agents, comprising a  $\beta$  1-4 polyuronic acid or chitosan.

It is also an object of the invention to provide a novel method for producing *in vitro* cytokines, such as lymphokines and induce receptors to these cytokines.

It is also an object of the invention to produce *in vivo* cytokines for the treatment of various diseases such as cancer, immune deficiencies, autoimmune diseases and infectious bacterial and viral diseases.

It is also an object of the invention to use the  $\beta$  1-4 polyuronic acids or chitosan for the manufacture of a pharmaceutical composition for the stimulation of cytokine production in monocytes, of a diequatorially bound  $\beta$  1-4-polyuronic acid or chitosan.

The pharmaceutical compositions may include the  $\beta$  1-4 polyuronic acids or chitosans formulated in any suitable manner for administration to a patient, either alone or in admixture with suitable pharmaceutical carriers or adjuvants.

It is especially preferred to prepare the compositions for systemic therapy as parenteral formulations, but other forms of administration such as oral administration are also envisaged.

Suitable parental preparations of the compositions according to this invention are injection of infusion solution comprising non-toxic, inert, liquid carriers or diluents.

Suitable oral preparation of the compounds according to this invention are tablets or capsules comprising non-toxic inert solid carriers, diluents or other acceptable additives.

The preparations can contain inert or pharma-codynamically active additives. Furthermore, substances usually used as preserving and/or stabilizing agents, salts for varying the osmotic pressure, buffers and other additives may also be present.

The dosages in which the preparations are administered can vary according to the indication, the mode of use and the route of administration. The systemic therapy for an adult average patient in need of treatment achievable by stimulation of cytokin production in monocytes will be about 0.1-100mg/kg body weight/injection by parenteral administration.

## BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 is a graph showing the production of TNF- $\alpha$  from monocytes stimulated with poly-M (circle), LPS (triangle), SG (star), C60XY (square) and chitosan (diamond). Production of TNF- $\alpha$  from unstimulated monocytes was less than 0.1 ng/ml

Figure 2 is a graph showing the production of TNF- $\alpha$  from monocytes stimulated with samples of poly-M (a) and chitosan (b) with different molecular weights. Production of TNF- $\alpha$  from unstimulated monocytes was less than 0.08 ng/ml.

Figure 3 is a graph over time showing the effect of an injection of 250 mg C60XY into mice on the production of both TNF- $\alpha$  and IL-6. Background levels were less than 0.003 ng/ml of serum TNF- $\alpha$  and less than 0.019 ng/ml serum IL-6.

Figure 4 is a graph showing the inhibition of TNF- $\alpha$  (a), IL-6 (b) and IL-1 (c) production from monocytes stimulated with M-blocks without (open square) and with addition of 1 mg/ml G-blocks (filled square). Production of cytokines from unstimulated monocytes and monocytes stimulated with 1 mg/ml G-blocks were less than 0.01 ng/ml.

Figure 5 is a schematic representation of a part of the structures of the different polysaccharides used in this invention.

## DETAILED DESCRIPTION OF THE INVENTION

As used herein, the terms and expressions "leukocyte derived cytokines," lymphokines and cytokines are to be given a broad meaning. Specifically, the term "leukocyte" as used herein means mammalian cells of granulocytic and lymphocytic lineage. Examples of leukocyte cells are polymorphonuclear leukocytes, such as neutrophils, and mononuclear phagocytes, such as monocytes and macrophages and lymphocytes.

The terms "cytokine" and "lymphokines" as used herein mean a secretory product of a leukocyte, and in particular a non-antibody protein released by a leukocyte on contact with antigen and which acts as an inter-cellular mediator of immune response. Examples of cytokines that are within the scope of this invention are chemotactic factors, factors promoting replication of lymphocytes, factors inhibiting replication of lymphocytes, factors affecting macrophage adherence, factors affecting enzyme secretion by macrophages, and factors that mediate secretion of oxidizing agents, such as oxygen, superoxide, hydrogen peroxide and hydroxyl radical. Specifically, TNF, IL-1 and IL-6 have been tested as being representative of the various cytokines which may be found.

## MATERIALS AND METHODS

### Polysaccharides

The monomer composition and sequential arrangement as well as the degree of polymerization ( $DP_n$ ) of the different polysaccharides used in the present invention were analyzed by <sup>1</sup>H-n.m.r. spectroscopy on a Bruker 400 WM spectrometer as described previously by Grasdalen et al. ("A p.m.r. studie of composition and sequence of uronate residues in alginate"; Carbohydr Res 1979; 68:23); see also, Grasdalen H. 1983. High-field <sup>1</sup>H-n.m.r. spectroscopy of alginate: sequential structure and linkage conformation. Carbohydr. Res., 118:255).

M-blocks consisting of more than 95% D-mannuronic acid (D-ManA) were isolated from the intracellular substance of *Ascophyllum nodosum* fruiting bodies as described by Haug et al. ("Correlation between chemical structure and physical properties of alginates" *Acta chem scand* 1967:21:768). The M-blocks had a  $DP_n$ =30-35.

G-blocks consisting of more than 90% L-guluronic acid (L-GulA) and with a  $DP_n$ =25-30 were isolated from the alga *Laminaria digitata* by the method described by Haug et al. ("Studies on the sequence of ionic acid residues in alginic acid 1967:21:691).

A high molecular weight homopolymeric  $\beta$ 1-4 linked D-ManA (poly-M) was isolated from liquid cultures of *Pseudomonas aeruginosa* DE 127 grown at low temperatures as described previously (Skjåk-Bræk G., Grasdalen H. and B. Larsen. 1986. Monomer sequence and acetylation pattern in some bacterial alginates. Carbohydr. Res., 154:239). The weight average molecular weight ( $M_w$ ) was estimated from intrinsic viscosity measurement using the Mark-Howink Sakurada relation (Harding S., Vårum K.M., Stokke B.T. and O. Smidsrød. 1990. Molecular weight determination of polysaccharide. Advances in Carbohydrate Chemistry. Vol 1. JAI press., in press.). Aqueous solutions of sodium chloride (0.1M) were used at 20°C in a Ubbelohde suspended capillary viscometer with an automatic dilution viscosity system (Scott-Geräte). From this high molecular weight poly-M, samples with different chain length were prepared by partial hydrolysis for 10-120 min. at 100°C and pH 5.4.

The  $\beta$ 1-4 linked glucuronic acid (D-GlcA) (C60XY), prepared by oxidation of cellulose at position C-6 (Painter T.J., Cesaro A., Delben F. and S. Paoletti. 1985. New glucuronoglucan obtained by oxidation of amylose at position 6. Carbohydr. Res., 140:61), was provided by Dr. A. Cesaro, Trieste, Italy. The molecular weight ( $M_w$ ) was estimated by viscometry to be 60,000; and the degree of oxidation (94%) was determined by potentiometric titration.

Chitin was isolated (Hacman R.H. 1954. Studies on chitin. I. Enzymic degradation of chitin and chitin esters. Aust. J. Biol. Sci., 7:168) from shrimp shell and milled in a hammer mill to pass through a 0.5 mm sieve. N-deacetylation of chitin under homogeneous conditions involved the procedure of Sannan et al. (Sannan T., Kurita K. and Y. Iwakura. 1976. Studies on Chitin. 2. Effect of Deacetylation on Solubility. Macromol. Chem., 177:3589). The degree of deacetylation (40%) was determined by high-field N.M.R.-spectroscopy (Vårn K.M., Anthonsen M.W., Grasdalen H. and O. Smidsrød. 1990. N.m.r. Spectroscopy of partially N-deacetylated Chitins (Chitosans). I. Determination of the Degree of N-acetylation and the Distribution of N-acetyl Groups. Carbohydr. Res., In Press). Chitosan was dissolved in acetic acid (2% w/v) by gentle shaking overnight at ambient temperature. Chitosan of different molecular weight were prepared by adding a known amount (10-100mg) of solid  $\text{NaNO}_2$ , each solution was stored in the dark at room temperature overnight, and finally neutralized with NaOH. The degraded chitosans were conventionally reduced with sodium-borohydride (250 mg). The pH of the solutions were adjusted to 5 with HCl, the solutions were dialyzed against 0.2M NaCl and distilled water, and finally lyophilized. This converted the chitosans into the chloride salt which is readily soluble in water. The number average molecular weight ( $M_n$ ) was determined by osmotic pressure measurements of solutions of chitosan in a Knauer Membrane Osmometer with a Sartorius SM 11736 cellulose-acetate membrane. Chitosan concentrations of 0.1 - 0.8 % (w/v) were used.

Scleroglucan (SG), a  $\beta$ 1-3 linked D-glucose with single  $\beta$ 1-6 linked glycopyranose residues on every third monomer with a molecular weight of 384,000 ( $M_w$ ), was provided by Dr. B.T. Stokke, Institute of Physics, NTH, University of Trondheim, Trondheim, Norway.

*E. coli* derived LPS (Sigma) (strain 026:B6) was used in these experiments.

Endotoxin contamination in the different polysaccharides was measured by the LAL assay (Kabi vitrum, Stockholm, Sweden). The levels of endotoxin were as follows:

- 1  $\mu$ g of C60XY contains 40 pg
- 1 mg of chitosan contains 1.3 pg
- 1 mg of SG contains 72 pg.

#### Monocyte cultivation

Monocytes were isolated from human A<sup>+</sup> blood buffy coat (The Bloodbank, University of Trondheim, Norway) as described by Bøyum (Bøyum AM. 1976. Separation of monocytes and lymphocytes. Scan. J. Immunol., 5:9). Monocytes in 24 well culture plates (Costar, Cambridge, MA, USA) were cultured in complete medium consisting of RPMI 1640 (Gibco, Paisley, U.K.) with 1% glutamine, 40  $\mu$ g/ml garamycin and 25% A<sup>+</sup> serum (The Bloodbank, University of Trondheim).

The different polysaccharides and LPS were dissolved in PBS and sterile filtered through 0.2  $\mu$ m filter (Nuclepore, Pleasanton, CA). The polysaccharide and LPS solutions were diluted in complete medium and added to the monocytes for 16-24 hours before the supernatants were harvested. Some monocyte cultures were preincubated with recombinant (r) human IFN- $\gamma$  (rIFN- $\gamma$ , Genentech, Inc. South San Francisco, CA) for 30. min. before the polysaccharides and LPS were added.

#### Animals

8-11 week old female CB<sub>6</sub>F<sub>1</sub> mice were purchased from Bomholdt Gård Breeding Research Center, Ry, Denmark. The mice were allowed access to ordinary chow and water ad libitum and weighed 20-22 g on the day of the study.

#### In Vivo Experiment

For cytokine induction mice were injected iv. with 0.2 ml of sterile saline containing 250  $\mu$ g of C60XY. At different time points after injection three mice were sacrificed and bled for separation of serum. Individual serum samples were kept in sterile tubes at -20°C until tested for TNF- $\alpha$  and IL-6 activities.

Assay for detection of TNF- $\alpha$  in supernatants from monocytes:

TNF- $\alpha$  activity was determined by its cytotoxic effect on the fibrosarcoma cell line WEHI 164 clone 13, as described (Espevik T. and J. Nissen-Meyer. 1986. A highly sensitive cell line, WEHI 164 clone 13, for measuring cytotoxic factor/tumor necrosis factor from human monocytes. J. Immunol. Methods, 95:99). Dilutions of rTNF- $\alpha$  (rTNF- $\alpha$  (human) and rMuTNF- $\alpha$ , Genentech) were included as a standard. The TNF- $\alpha$  specificity of the assay was verified by using a monoclonal antibody against rTNF- $\alpha$  which completely neutralized the recorded activity (data not shown). Results are presented as ng/ml  $\pm$  S.E. of triplicate determinations.

Assay for detection of IL-1 in supernatants from monocytes:

IL-1 activity was determined by a two stage assay. The first stage involves the mouse thymocyte EL4 NOB-I cell line which produces high concentrations of Interleukin-2 (IL-2) in response to human IL-1, as described by Gaering et al. (Gearing A.J.H., Bird C.R., Bristow A., Poole S. and R. Thorpe. 1987. "A simple sensitive bioassay for Interleukin-1 which is unresponsive to  $10^3$  U/ml of Interleukin-2." J. Immunol. Methods, 99:7). Dilutions of rIL-1 $\beta$  (Glaxo, Geneva Switzerland) were included as standard. After incubation in 37°C, 5% CO<sub>2</sub> atm. for 24 hours 100  $\mu$ l of the supernatants were transferred into a replicate 96-well microplate. The second stage in this assay involves the IL-2 dependent mouse T cell line HT-2 as described by Mosmann, T. (Mosmann T.R., Chervinski H., Bond M.W., Giedlin M.A. and R.L. Coffman. 1986. Two types of murine helper-T cell clone. 1. Definition according to profiles of lymphokine activities and secreted proteins. J. Immunol., 136:2348). One hundred  $\mu$ l of HT-2 suspension ( $1.5 \times 10^5$  cells/ml) were added to each well and incubated for an additional 24 hours. The IL-1 activity was completely neutralized by two polyclonal antibodies against rIL-1 $\beta$  and rIL-1 $\alpha$  (gift from Dr. A. Shaw, Glaxo, Geneva, Switzerland), (data not shown). Results are presented in Table 2 as ng/ml  $\pm$  S.E. of triplicate determinations.

Assay for detection of IL-6 in supernatants from monocytes:

IL-6 activity was determined by the IL-6 dependent mouse hybridoma cell line B.13.29 clone 9, as described by Aarden et al. (Aarden L.A., De Groot E.R., Schaap O.L. and P.M. Lansdorp. 1987. Production of hybridoma growth factor by monocytes. Eur. J. Immunol., 17:1411). Dilutions of the supernatants and rIL-6 (Brakenhoff J.P.J., De Groot E.R., Evers R.F., Pannekoek H. and L.A. Aarden. 1987. Molecular cloning and expression of hybridoma growth factor in *Escherichia coli*. J. Immunol., 139:4116) as a standard, were incubated in a 96-well microplate together with cells ( $5 \times 10^4$  cells/ml) for 72 hours. The plates were harvested and the IL-6 activity was determined colorimetrically (see below). The IL-6 activity was completely neutralized by a polyclonal antibody against rIL-6 (gift from Dr. W. Fiers, University of Ghent, Belgium), (data not shown). Results are presented in Table 2 as ng/ml  $\pm$  S.E. of triplicate determinations.

MTT-assay (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide):

Viability in the assays for TNF- $\alpha$ , IL-1 and IL-6 were measured in a colorimetric assay for growth and survival by using a tetrazolium salt as described by Mosmann (Mosmann T. 1983. Rapid colorimetric assay for cellular growth and survival: Application to proliferation and cytotoxicity assays. J. Immunol. Methods, 65:55).

Results

In the present invention we have isolated polymers of  $\beta$  1-4 linked D-ManA (poly-M) from *Pseudomonas aeruginosa* and compared its TNF- $\alpha$  inducing ability with other  $\beta$  1-4 and  $\beta$  1-3 linked polysaccharides. Fig. 1 shows that poly-M is approximately equally potent as LPS, around 100 times more potent than  $\beta$  1-3 linked D-glucose (SG), 500 times more potent than cellulose oxidized in C-6 position (C60XY) and 10,000 times more potent than chitosan in their respective abilities to induce production of the cytokines.

Since the poly-M was isolated and purified from liquid cultures of a Gram negative bacteria, the possibility existed that the high TNF- $\alpha$  stimulating activity could be due to LPS contamination. However, LPS levels in the poly-M preparation was less than 1 ng in 1  $\mu$ g of poly-M. Since *E. coli* LPS at 1 ng/ml did not stimulate TNF- $\alpha$  production from monocytes (data not shown) it was shown that LPS contamination was not the cause of the stimulating activity. Furthermore, reduction of the LPS content in the poly-M preparation by using a polymyxin B column and a dialyzable surfactant, octyl- $\beta$ -D-glycopyranoside (Karplus T.E., Ulevitch R.J. and C.B. Wilson. 1987. A new method for reduction of endotoxin contamination from protein solutions. J. Immunol. Methods, 105:211) did not reduce its TNF- $\alpha$  stimulating ability (data not shown). Consequently, it is unlikely that the

stimulatory effect of poly-M at 0.1 µg/ml and 0.01 µg/ml is due to LPS contamination.

The prior art established that in order for the  $\beta$ 1-3 glucans to have optimal antitumor activity the molecular weight must be higher than 50,000 (Kojima T., Tabata K., Itoh W. and T. Yanaki. 1986. Molecular Weight Dependence of the Antitumor Activity of Scizophyllan. *Agric. Biol. Chem.*, 50(1):231). It was therefore of interest to examine the relationship between the molecular weight of  $\beta$ 1-4 linked polysaccharides and their TNF- $\alpha$  inducing capability. The results shown in Fig. 2 a and b demonstrate that the molecular weight must be higher than 20,000 and 50,000 for chitosan and poly-M, respectively, in order to obtain maximal stimulatory effect.

Activation of monocytes with IFN- $\gamma$  increases their ability to produce TNF- $\alpha$  in response to LPS (Nissen-Meyer J., Hofslie E., Espevik T and R. Austgulen. 1988. Involvement of Tumor Necrosis Factor in cytotoxicity Mediated by Human Monocytes. *Nat. Immun. Cell Growth Regul.*, 7:266). We therefore examined whether IFN- $\gamma$  could increase the TNF- $\alpha$  production from polysaccharide stimulated monocytes. Table 2 shows that IFN- $\gamma$  increased the TNF- $\alpha$  production from chitosan, C60XY, SG and poly-M stimulated monocytes in a similar way as for LPS. Therefore, the present invention includes the use of IFN- $\gamma$  in conjunction with the polysaccharides of the present invention in order to increase the stimulation of the cytokine production.

Since polysaccharides consisting of  $\beta$ 1-4 linked poly uronic acids were very potent in stimulating TNF- $\alpha$  production *in vitro*, we tested whether C60XY had immunostimulating activities in mice. As illustrated in Fig. 3, the injection of 250 µg C60XY into mice led to the rapid production of both TNF- $\alpha$  and IL-6. Although measurable levels of IL-6, but not TNF- $\alpha$ , could be detected 30 min. after injection, the peak level for both cytokines was attained 60 min. after C60XY administration. The activities of both TNF- $\alpha$  and IL-6 were eliminated rapidly reaching baseline levels within 60 minutes of peak time.

In summary, the compositions employed in the present invention are capable of stimulating the effects of leukocyte derived cytokines, such as TNF, IL-1 and IL-6, on phagocytes, such as polymorphonuclear leukocytes.

Because IL-1, IL-6, TNF, and other leukocyte derived cytokines have been implicated in such a wide variety of mammalian conditions, this invention has a similarly broad scope of application. Among the conditions that can be treated or alleviated by the inhibition or stimulation of IL-1, IL-6, TNF, and other leukocyte derived cytokines are: sepsis, septic shock, endotoxic shock, gram negative sepsis, toxic shock syndrome, adult respiratory distress, fever and myalgias due to infection (i.e. influenza), cachexia secondary to infection or malignancy, cachexia secondary to AIDS rheumatoid arthritis, gouty arthritis, osteoporosis, keloid formation, scar tissue formation, decreased appetite, Crohn's disease, ulcerative colitis, fever due to central nervous system bleeding, glomerulonephritis, multiple sclerosis, Creutzfeld-Jacob disease, adverse reactions to dialysis and psoriasis.

By reference to the specific cause of the disease condition, the more generic term 'trauma' can be used. The term 'trauma' refers broadly to cellular attack by foreign bodies and physical injury of cells. Included among foreign bodies are microorganisms, particulate matter, chemical agents, and the like. Included among physical injuries are mechanical injuries, such as abrasions, lacerations, contusions, wounds, and the like; thermal injuries, such as those resulting from excessive heat or cold; electrical injuries, such as those caused by contact with sources of electrical potential; and radiation damage caused, for example, by prolonged, extensive exposure to infrared, ultraviolet or ionizing radiations.

Microorganisms included among the foreign bodies that can elicit a biological response are bacilli, fungi and yeast, viruses, parasites, and the like. Representative bacilli are: *Actinomyces* spp.; *Bacteroides* spp.; *Corynebacterium* spp.; *Enterobacteriaceae*; *Enterococcus*; *Haemophilus* spp.; *Micrococcus* spp.; *Neisseria* spp.; *Staphylococcus aureus*; *Streptococcus pneumoniae*; *E. coli*; *Clostridium* spp.; *Streptococcus agalactiae*; *Bacillus* spp.; *H. influenzae*; *Moraxella* spp.; *Mycobacteria* spp.; *Pseudomonas aeruginosa*; *Vibrio* spp.; and *Mycoplasma*.

Representative fungi and yeast that are capable of eliciting a biological response are: *Microsporum*; *Blasatomyces*; *Histoplasma*; *Aspergillus*; *Cryptococcus*; *Candida* spp.; *Coccidioides*; and *Candida albicans*.

Representative viruses are: Rhinovirus; Parainfluenza; Enterovirus; Influenza; Smallpox and vaccinia; Herpes simplex; Measles; Rubella; Arbovirus (Western, Eastern and Venezuelan equine encephalitis, and California encephalitis); Rabies; Colorado tick fever; Yellow fever; Dengue; Hepatitis Virus B (HB Ag); Hepatitis Virus A (HAV); and Human Immunodeficiency Virus (HIV).

Representative parasites that can elicit a response are: *Trypanosoma cruzi*; *Entamoeba histolytica*; *Leishmania brasiliensis*; *Leishmania tropica*; *Leishmania donovani*; *Toxoplasma gondii*; *Plasmodium falciparum*; *Trypanosoma rhodesiense*; *Loa loa*; *Trichomonas hominis*; *Schistosoma japonicum*; *Schistosoma mansoni*; and *Fasciola hepatica*.

Thus, it has been demonstrated that polymers of mannuronic acid are a very potent stimulator of TNF- $\alpha$  production from human monocytes. The high TNF- $\alpha$  inducing ability of poly-M was not restricted to poly-M produced by *Ps. udomonas aeruginosa*, as similar potency was found for poly-M isolated from liquid cultures of

Pseudomonas syringae by phaseolicola (Gross M. and K. Rudolph. 1983. Studies on the extracellular polysaccharides (EPS) produced *in vitro* by Pseudomonas phaseolicola. J. Phytopathology, 118:276 (unpublished data)). Poly-M is 100 times more potent than SG, 500 times more potent than C60XY and 10,000 more potent than chitosan. It has previously been reported that the anti-tumor effects of  $\beta$ 1-3 glucans are optimal at a molecular weight of over 50,000 (Kojima T., Tabata K., Itoh W. and T. Yanaki. 1986. Molecular Weight Dependence of the Antitumor Activity of Scizophyllan. Agric. Biol. Chem., 50(1):231) and that the anti-tumor effect is mediated by cytokines (Seljelid R., Figenschau Y., Bøgwald J., Rasmussen L.T. and R. Austgulen. 1989. Evidence that Tumour Necrosis Induced by Aminated  $\beta$ 1-3D Polyglucose is Mediated by a Concerted Action of Local and Systemic Cytokines. Scand. J. Immunol., 30:687). Our data indicate that maximal production of TNF- $\alpha$  occurred when the molecular weight of poly-M and chitosan was above 50,000 and 20,000 respectively. The difference in the potency to induce TNF- $\alpha$  production may be related to the polysaccharide structure as the molecular weight of the polysaccharides used was higher than 50,000 (Table 1). From Fig. 5 it can be seen that Poly-M is a homopolymer composed of  $\beta$ 1-4 linked D-ManA while chitosan is composed of randomly distributed units of  $\beta$ 1-4 linked N-acetylglucosamine (D-GlcNAc) and N-glucosamine (D-GlcN) (Vårum K.M., Anthonsen M.W., Grasdalen H. and O. Smidsrød. 1990. N.m.r. Spectroscopy of Partially N-deacetylated Chitins (Chitosans). I. Determination of the Degree of N-acetylation and the Distribution of N-acetyl Groups. Carbohydr. Res., In Press). The three dimensional (3D) structure of C60XY and poly-M is similar, but the sequences of  $\beta$ 1-4 linked D-guluronic acid (D-GlcA) in C60XY are broken by  $\beta$ 1-4 linked D-glucose (D-Glc) (6%). The homopolymeric sequences of  $\beta$ 1-4 linked uronic acids will consequently be shorter in C60XY than in poly-M. One possibility is that the high TNF- $\alpha$  inducing ability of poly-M compared to C60XY is caused by the longer homopolymeric  $\beta$ 1-4 linked uronic acid blocks. Additional structural similarities between the TNF- $\alpha$  stimulating polysaccharides poly-M, chitosan and C60XY are equal length between the monomers along the chains due to di-equatorial (di-eq) glycosidical bindings between the monomers (Fig. 5). These similarities in 3D structure are probably important for the TNF- $\alpha$  stimulating activity of poly-M, chitosan and C60XY.

The bacteria Pseudomonas aeruginosa is found in most patients with cystic fibrosis and colonization is associated with pulmonary damage (Simpson J.A., Smith S.E. and Dean R.T. 1988. Alginate Inhibition of Uptake of Pseudomonas aeruginosa by Macrophages. J. Gen. Microbiol., 134:29). The facts that high molecular (viscous) poly-M is produced by Pseudomonas aeruginosa, that poly-M is a very potent cytokine stimulator and that no enzyme in the human body is known to degrade poly-M could explain some of the difficulties in treating this infection in man.

Injection of 250  $\mu$ g C60XY in mice resulted in the release of TNF- $\alpha$  and IL-6 into the circulation. The serum concentrations of TNF- $\alpha$  and IL-6 from mice injected with C60XY are not due to LPS contamination as 1  $\mu$ g C60XY contained only 40 pg endotoxin, and injection of 10 ng LPS resulted in less than 27 pg/ml TNF- $\alpha$  and less than 21.5 ng/ml IL-6 in serum. In addition Shalaby et al. (Shalaby R.M., Waage A., Aarden L. and T. Espevik. 1989. Endotoxin, Tumor Necrosis Factor- $\alpha$  and Interleukin 1 Induce Interleukin 6 Production *in vivo*. Clinic. Immunol. and Immunopath. 53 488) have shown that the kinetics for serum TNF- $\alpha$  are different from the kinetics for serum IL-6 after LPS injection into mice. They found that peak serum IL-6 concentration was attained after 2 hr of LPS injection into mice, while the peak for TNF- $\alpha$  is attained after 1 hr. In this experiment the peak level for both cytokines was attained 1 hr after C60XY administration. These data indicate that di-eq  $\beta$ 1-4 linked poly uronic acids have potent immune stimulating activities *in vivo*.

Previously we have found that G-blocks are ineffective in stimulating monocytes to produce cytokines. Otterlei M., Østgaard K., Skjåk-Bræk G., Smidsrød O. and T. Espevik. 1991. Induction of cytokine production from human monocytes stimulated with alginate, J. of Immunotherapy, in press. Poly-M and C60XY have similar polysaccharide structure. Furthermore, as discussed above, the only structural difference between poly-M and M-blocks is the length of the homopolymeric D-ManA sequence (Fig. 5). In the M-blocks this sequence is broken up by L-GulA residues (5%) giving an average number of 5-7 consecutive D-ManA residues. The glycosidic linkages in M-blocks are di-eq with a monomer length 5.17 Å in the fully stretched form (Atkins E.D.T., Mackie E.E. and F.E. Smolko. 1970. Crystalline structure of alginic acid. Nature 225:626), but when a D-ManA is linked to a L-GulA instead of D-ManA the glycosidic linkage is an equatorial-axial (eq-ax) nature (Fig. 5). The length between the monomers in an eq-ax binding is shorter than in a di-eq binding. Since D-ManA and L-GulA have almost identical structures (different only in the configuration around C-5) the large difference between M- and G-blocks ability to stimulate cytokine production must be due to the difference in the conformation they adapt as part of a homopolymeric structure (see Fig. 5).

The G-blocks have mainly di-axially (di-ax) glycosidic linkages which are even shorter (4.36Å) than the eq-ax binding (Atkins E.D.T., Mackie E.E. and F.E. Smolko. 1970. Crystalline structure of alginic acid. Nature 225:626). G-blocks will also contain some ax-eq linkages between L-GulA and D-ManA (10%). The G-blocks are more compact than M-blocks, C60XY and poly-M but the chains have all two folded screw axis in the solid state and statistical mechanical calculations has shown that this linkage conformation is also highly populated



in solutions (Smidsrød O., Haug A. and S. Whittington. 1972. The molecular basis for some physical properties of polyuronides. Acta. Chem. Scand. 26:2563; Smidsrød O., Glover R.M. and S.G. Whittington. 1973. The relative extension of alginates having different chemical compositions. Carbohydr. Res. 27: 107).

TABLE 1

POLYSACCHARIDE	LINKAGE	MW/DP <sub>n</sub>	MONOMER COMPOSITION
Poly-M	β1-4	1270,000 (M <sub>w</sub> )	100% D-mannuronic acid
M-blocks	β1-4	DP <sub>n</sub> = 30-35	95% D-mannuronic acid, 5% L-guluronic acid
C6OXY	β1-4	60,000 (M <sub>w</sub> )	94% D-glucuronic acid, 6% D-glucose
Chitosan	β1-4	1100,000 (M <sub>n</sub> )	60% N-acetylglucosamine 40% N-glucosamine
G-blocks	α1-4	DP <sub>n</sub> = 25-30	90% L-guluronic acid 10% D-mannuronic acid
SG	β1-3	384,000 (M <sub>w</sub> )	100% D-glucose

<sup>1</sup> Samples used in Fig. 1

TABLE 2: Effects of IFN-γ on polysaccharide induced TNF-α [ng/ml] production from human monocytes.

Addition	Concentrations (μg/ml)	TNF-α [ng/ml]	
		-IFN-γ	+IFN-γ (1000 U/ml)
Medium	---	<0.1	<0.1
Chitosan	100	7.0 ± 0.4	32.7 ± 7.8
C6OXY	100	34.2 ± 3.6	68.9 ± 11.1
SCLE-1	1	2.9 ± 0.5	10.3 ± 2.3
Poly-M	0.1	45.8 ± 1.7	93.5 ± 19.7
LPS	0.1	23.7 ± 3.9	47.8 ± 10.1

## Claims

1. Pharmaceutical composition comprising a diequatorially bound  $\beta$  1-4-polyuronic acid or chitosan.
- 5 2. Pharmaceutical composition according to Claim 1 wherein said diequatorial bound  $\beta$  1-4 polyuronic compounds are selected from the group consisting of polymers of different size of  $\beta$  1-4 linked D-mannuronic acid and cellulose oxidized in C-6 position (C6OXY).
- 10 3. Pharmaceutical composition according to Claim 1 wherein said diequatorial bound  $\beta$  1-4 polyuronic compound is  $\beta$ 1-4 linked D-ManA.
4. Pharmaceutical composition according to Claim 3 wherein said  $\beta$  1-4 linked D-ManA has a molecular weight greater than 50,000.
- 15 5. Pharmaceutical composition according to Claim 1 wherein said chitosan has a molecular weight greater than 20,000.
6. Pharmaceutical composition according to Claim 1 further comprising treating said monocytes with an effective amount of IFN- $\gamma$  prior to treatment with said diequatorial bound  $\beta$  1-4 polyuronic acid.
- 20 7. Use for the manufacture of a pharmaceutical composition for the stimulation of cytokine production in monocytes, of a diequatorially bound  $\beta$  1-4-polyuronic acid or chitosan.
8. A method of stimulating monocyte production of IL-1, IL-6 and TNF- $\alpha$  comprising treating said monocytes with an effective amount of a composition selected from diequatorial bound  $\beta$  1-4 polyuronic compounds selected from the group consisting of polymers of different size of  $\beta$  1-4 linked D-mannuronic acid and cellulose oxidized in C-6 position (C6OXY), and chitosans.
- 25 9. The methods of Claim 8 further comprising incubating said monocytes, in an effective amount of IFN- $\gamma$  prior to treatment with said diequatorial bound  $\beta$  1-4 polyuronic acid or chitosans.
- 30 10. A method of treating a bacterial, fungal or viral infection, or neoplasia comprising administration of a composition of Claim 1.
11. Method for in vivo or in vitro stimulation of monocytes for the production of cytokines.

Fig.1.

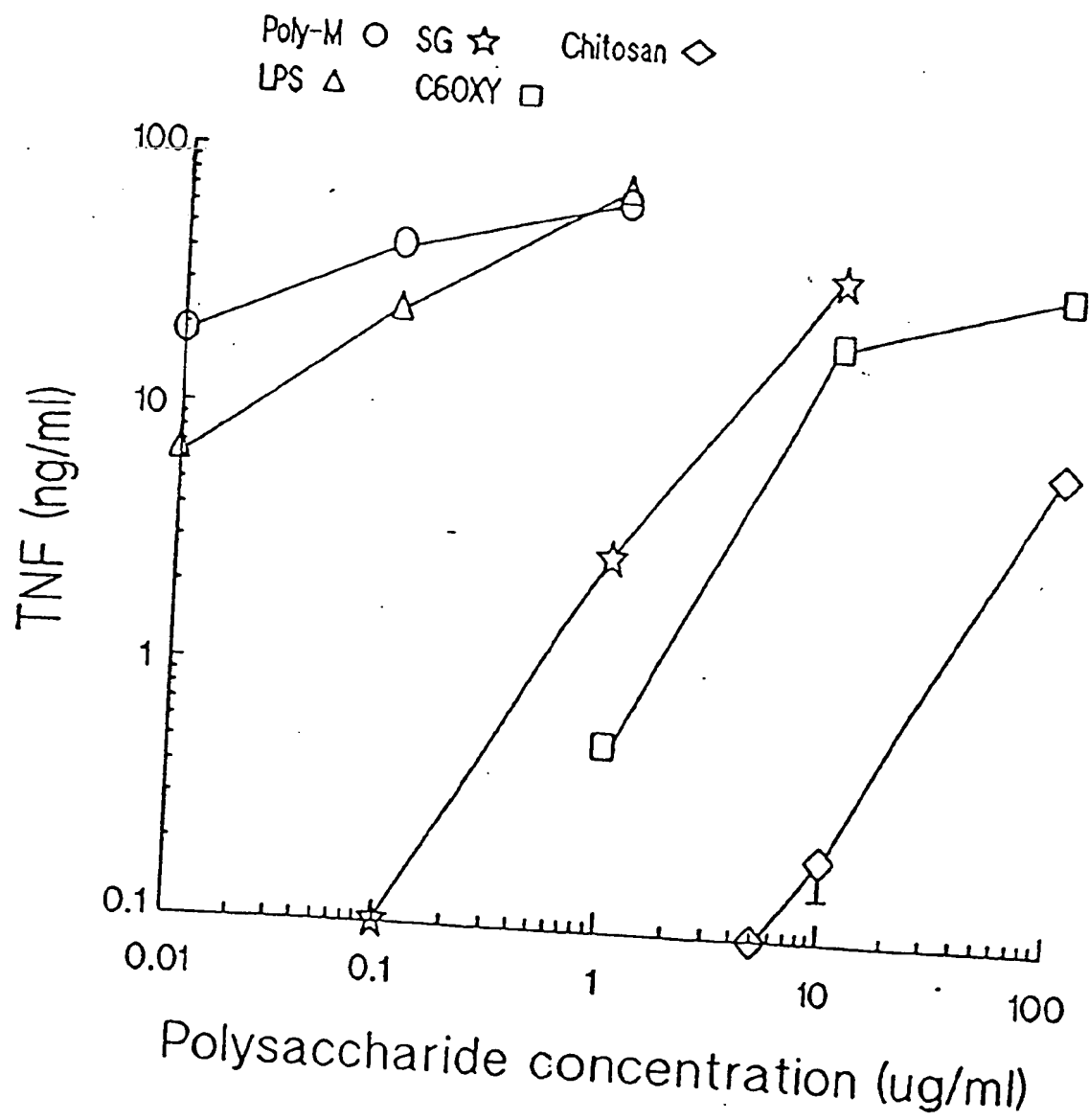


Fig. 2

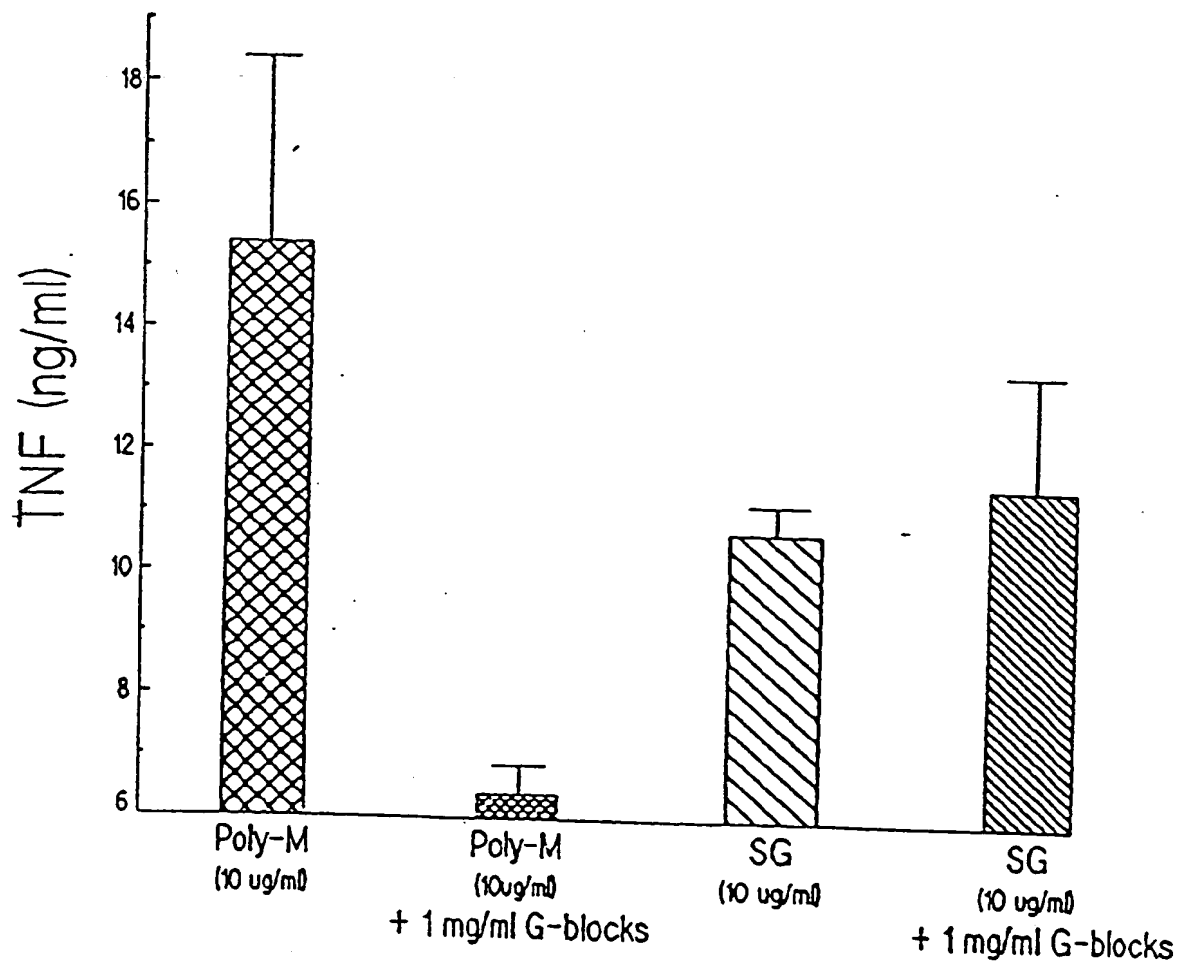


Fig. 3

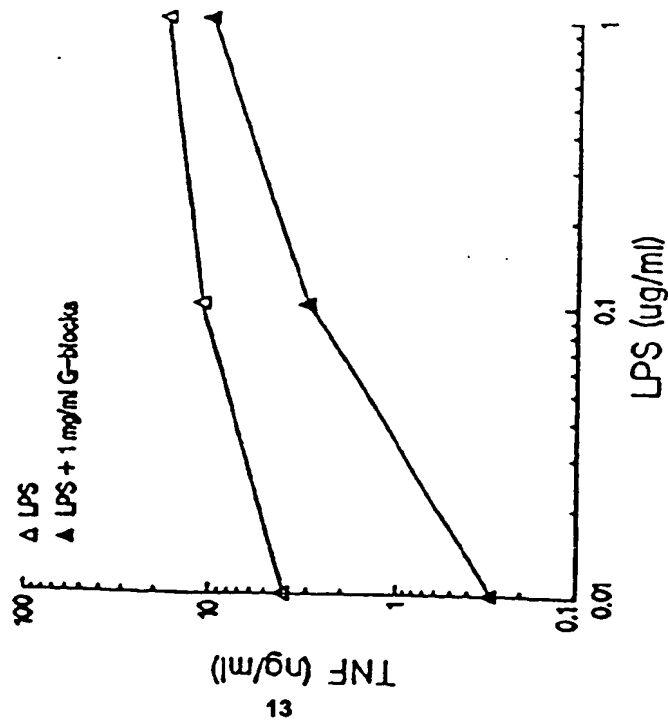


Figure 4

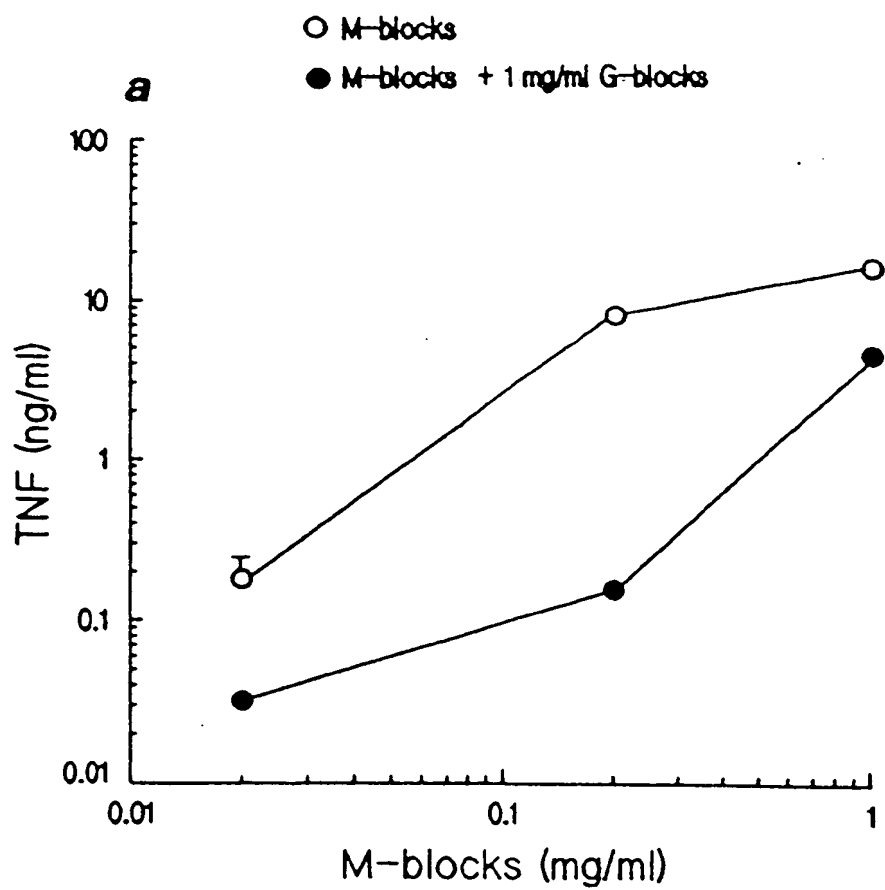


Figure 4 continued

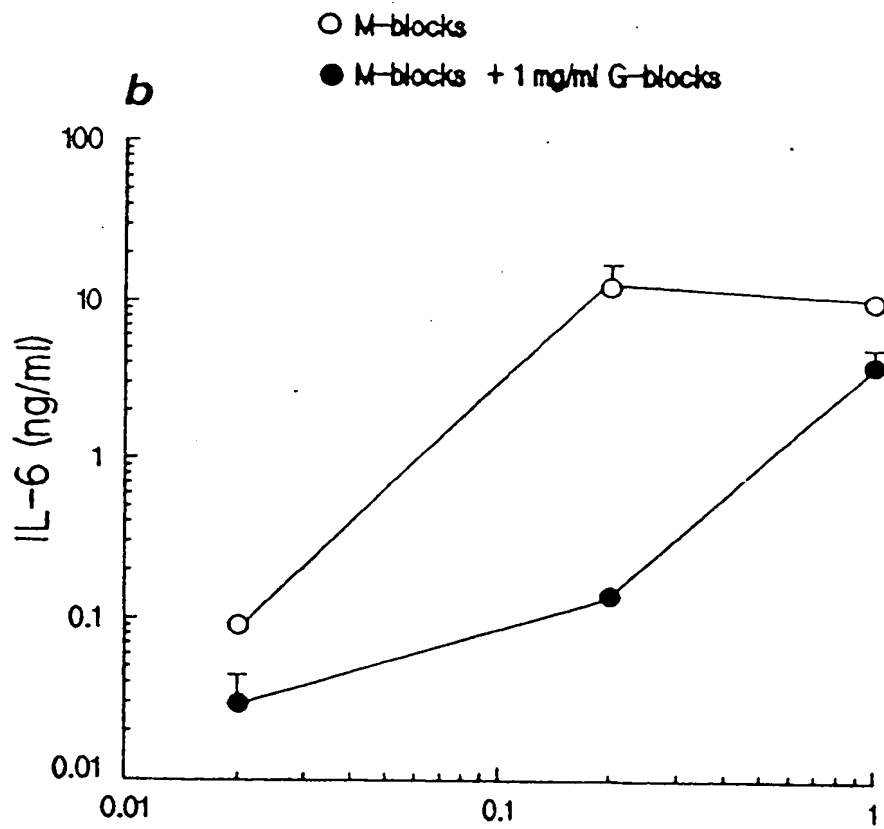


Figure 4 continued

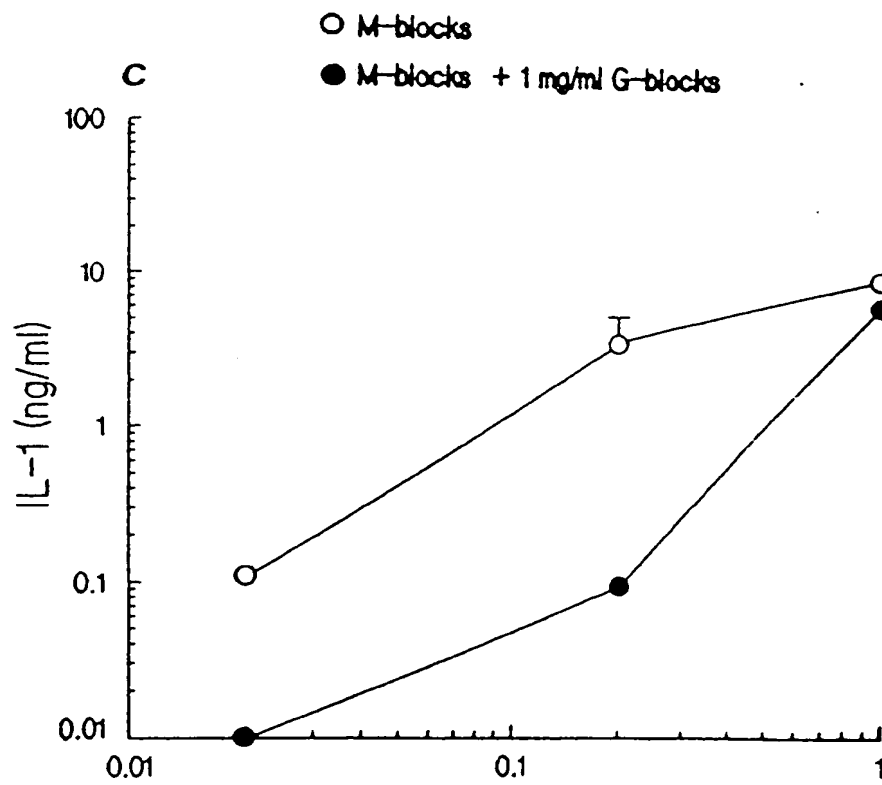
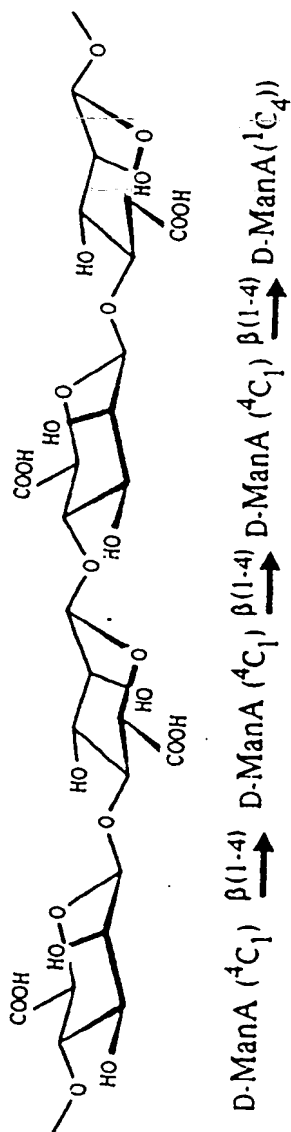




Figure 5

Poly-M



M-blocks

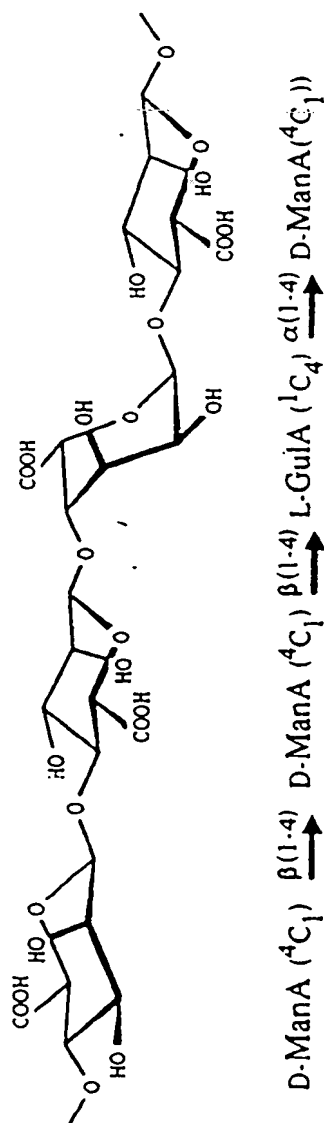
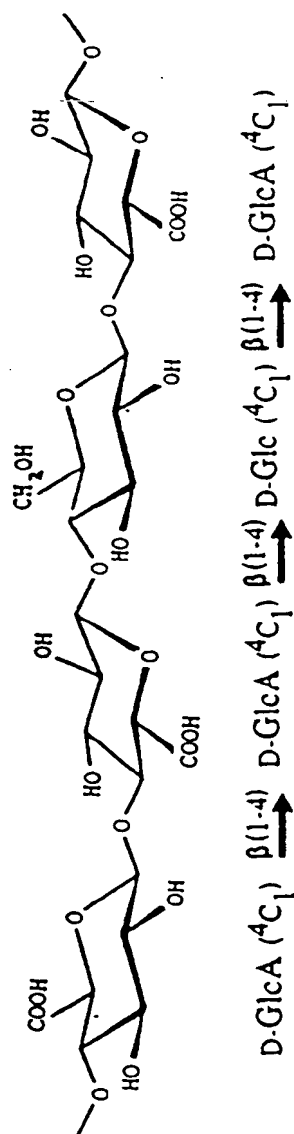


Figure 5 continued

C6OXY



Chitosan

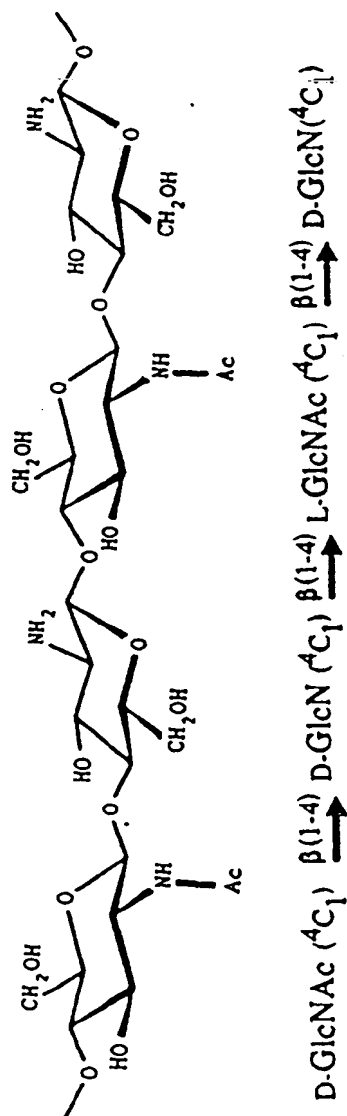
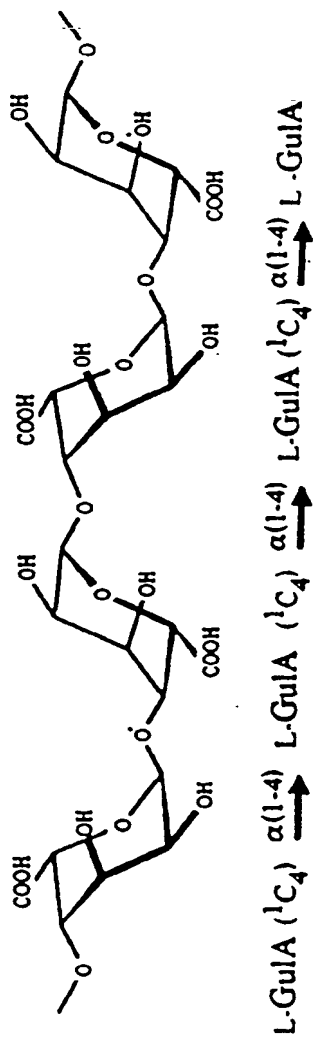


Figure 5 continued

G-blocks





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(54) **Diequitorially bound beta 1-4 polyuronates and use of same for cytokine stimulation.**

(57) The present invention is directed to a material and method for the stimulation of the production of cytokines. Several polysaccharides, including polymers of different size of  $\beta$ 1-4 linked D-mannuronic acid (poly-M and M-blocks), chitosan and cellulose oxidized in C-6 position (C6OXY) induce human monocytes to produce the cytokines. Preferably, the molecular weights of poly-M and chitosan are above 50,000 and 20,000 respectively. Pretreatment of the monocytes with IFN- $\gamma$  increases the cytokine production from monocytes stimulated with all polysaccharides tested. The subject polysaccharides worked *in vivo* and *in vitro*.

The present invention has therapeutic utility as vaccine adjuvants and components. Therapeutic compositions comprising biologically active quantities of the compositions of the present invention may be employed to potentiate antibody production in response to vaccine antigens. Anti-tumor, anti-bacteriological, anti-fungal and anti-viral effects may be expected.

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# EUROPEAN SEARCH REPORT

Application Number

EP 92 30 2499

DOCUMENTS CONSIDERED TO BE RELEVANT			
Category	Citation of document with indication, where appropriate, of relevant passages	Relevant to claim	CLASSIFICATION OF THE APPLICATION (Int. CL.5)
X	PATENT ABSTRACTS OF JAPAN, vol. 12, no. 426 (C-542)[3273], 10th November 1988; & JP-A-63 156 726 (LION CORP.) 29-06-1988 * Abstract *	1,5	A 61 K 31/73 A 61 K 31/715
X	PATENT ABSTRACTS OF JAPAN, vol. 10, no. 230 (C-365)[2286], 9th August 1986; & JP-A-61 64 701 (MEITO SANGYO K.K.) 03-04-1986 * Abstract *	1	
X	FILE WPI, accession no. 75-27072W [16], Derwent Publications Ltd, London, GB; & SU-A-430 857 (KHARKOV) 20-11-1974 * Abstract *	1,2	
P,X	REGIONAL IMMUNOLOGY, vol. 3, no. 6, 1991, pages 341-348, John Wiley & Sons, Inc., H. SECRIST et al.: "Induction of IL-4 and IL-6 synthesis in vitro: variation in signaling requirements and kinetics are dependent on the anatomic source of the responding mononuclear cells" * Summary *	11	TECHNICAL FIELDS SEARCHED (Int. CL.5) A 61 K
X	INT. J. IMMUNOPHARMACOL., vol. 12, no. 8, 1990, Pergamon Press, GB; J. CLOT et al.: "Effect in vitro of a bacterial extract (OM-89) on interleukin 1 and interleukin 2 production by peripheral blood mononuclear cells from healthy subjects and rheumatoid arthritis patients" * Abstract *	11	
The present search report has been drawn up for all claims			
Place of search THE HAGUE		Date of completion of the search 05-10-1992	Examiner KLAVER T.
<p><b>CATEGORY OF CITED DOCUMENTS</b></p> <p>X : particularly relevant if taken alone Y : particularly relevant if combined with another document of the same category A : technological background O : non-written disclosure P : intermediate document</p> <p>T : theory or principle underlying the invention E : earlier patent document, but published on, or after the filing date D : document cited in the application L : document cited for other reasons A : member of the same patent family, corresponding document</p>			



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### CLAIMS INCURRING FEES

The present European patent application comprised at the time of filing more than ten claims.

- ☐ All claims fees have been paid within the prescribed time limit. The present European search report has been drawn up for all claims.
- ☐ Only part of the claims fees have been paid within the prescribed time limit. The present European search report has been drawn up for the first ten claims and for those claims for which claims fees have been paid, namely claims:
- ☐ No claims fees have been paid within the prescribed time limit. The present European search report has been drawn up for the first ten claims.

### LACK OF UNITY OF INVENTION

The Search Division considers that the present European patent application does not comply with the first requirement of unity of invention and relates to several inventions or groups of inventions, namely:

see sheet -B-

- ☒ All further search fees have been paid within the fixed time limit. The present European search report has been drawn up for all claims.
- ☐ Only part of the further search fees have been paid within the fixed time limit. The present European search report has been drawn up for those parts of the European patent application which relate to the inventions in respect of which search fees have been paid, namely claims:
- ☐ None of the further search fees has been paid within the fixed time limit. The present European search report has been drawn up for those parts of the European patent application which relate to the invention first mentioned in the claims, namely claims:



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EP 92 30 2499

**LACK OF UNITY OF INVENTION    a posteriori**

The Search Division considers that the present European patent application does not comply with the requirement of unity of invention and relates to several inventions or groups of inventions, namely:

1. Claims: 1-6    Pharmaceutical compositions of chitosan or  $\beta$ 1-4-polyuronic acid
2. Claims: 7-11    Use of  $\beta$ -1-4-polyuronic acid or chitosan to stimulate cytokine production.